Transcriptional characteristics and differences in Arabidopsis stigmatic papilla cells pre- and post-pollination

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Abstract: Pollination is an important early step in sexual plant reproduction. In Arabidopsis thaliana, sequential pollination events, from pollen adhesion onto the stigma surface to pollen tube germination and elongation, occur on the stigmatic papilla cells. Following successful completion of these events, the pollen tube penetrates the stigma and finally fertilizes a female gametophyte. The pollination events are thought to be initiated and regulated by interactions between papilla cells and pollen. Here, we report the characterization of gene expression profiles of unpollinated (UP), compatible pollinated (CP) and incompatible pollinated (IP) papilla cells in A. thaliana. Based on cell type-specific transcriptome analysis from a combination of laser microdissection and RNA sequencing, 15,475, 17,360 and 16,918 genes were identified as expressed in UP, CP and IP papilla cells, respectively, and of these, 14,392 genes were present in all three data sets. Differentially expressed gene (DEG) analyses identified 147 and 71 genes up-regulated in CP and IP papilla cells, respectively, and 115 and 46 genes down-regulated. Gene Ontology and metabolic pathway analyses revealed that papilla cells play an active role as the female reproductive component in pollination, particularly in information exchange, signal transduction, internal physiological changes and external morphological modification. This study provides fundamental information on the molecular mechanisms involved in pollination in papilla cells, furthering our understanding of the reproductive role of papilla cells.

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Research Article

Title

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Running Title

Pollination transcriptome

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Abbreviations

differentially expressed genes, DEG; gene ontology, GO; Laser microdissection, LM; RNA sequencing, RNA-seq; self-incompatibility, SI; RNA integrity number, RIN

Abstract (less than 250 words)

Pollination is an important early step for sexual plant reproduction. In *Arabidopsis thaliana*, sequential pollination events, from pollen adhesion on stigma surface to pollen tube germination and elongation, occur on the stigmatic papilla cell, and then pollen tube exactly penetrates into the stigma and finally fertilizes with a female gametophyte. It has been considered that these pollination systems are initiated and regulated by interaction between papilla cell and pollen. Here, we report the characterization of gene expression profiles among non pollinated- (NP), compatible pollinated- (CP) and incompatible pollinated- (IP) papilla cells in *A. thaliana*. By the cell type-specific transcriptome analysis, from a combination of laser microdissection and RNA sequencing, 15,475, 17,360 and 16,918 genes were identified as the papilla cell expressed genes for NP, CP and IP papilla cells, respectively, and, among them, 14,392 genes were commonly found in all data sets. In differentially expressed genes (DEGs) analysis, 147 and 71 genes were up-regulated in CP and IP papilla cells, and 115 and 46 genes were down-regulated in CP and IP papilla cells. Through Gene Ontology and metabolic pathway analyses, it was revealed that papilla cells are active, as a female reproductive organ in pollination, particularly in information exchange, signal transduction, internal physiological change and external morphological modification. This study provides fundamental knowledge of molecular mechanisms on pollination in papilla cell, leading our understanding of the reproductive role of papilla cells.

Keywords

Arabidopsis thaliana, laser microdissection, papilla cell, pollination, RNA sequencing, transcriptome

Introduction

In angiosperm, sexual reproduction is a complex of various events and regulated by multiple processes for successful seed production. When mature pollen grains land on the stigma, adhesive interaction between pollen and stigma are initiated and stronger adhesion is established by forming a structure called the foot (Gaude and Dumas 1984, Dickinson 1995). After that, pollen is recognized whether it is accepted or rejected by pollen recognition system(s), such as self-incompatibility (SI) and cross-compatibility, and accepted pollen rapidly starts its hydration by receiving water and resources for germination and pollen tube elongation, from pistil. Pollen tube exactly penetrates, grows and arrives at vicinity of ovule through the stigma, style and ovarian transmitting tract, and tube tip invades synergid cells by pollen tube guidance signal (Cheung et al. 1995, Franklin-Tong 1999, Lord 2000, Shimizu and Okada 2000, Takeuchi and Higashiyama 2012). Finally, two sperm cells are released from the pollen tube tip and fertilize with an egg cell and central cell, respectively, termed double fertilization (Dickinson and Elleman 1994, Stephenson et al. 1997).

Pollination is an initial important step for successful sexual reproduction in plants. Brassicaceae species have dry type of stigma, and pollination events occur on the stigmatic surface cell, the papilla cell (Heslop-Harrison and Shivanna 1977). Papilla cell is a large single cell, having two layers of cell wall, cuticle and pectocellulosic layer (Elleman et al. 1992, Elleman et al. 1988). It has been considered that pollen recognition, pollen hydration

97 and pollen tube guidance to stigma were regulated by interaction between papilla cell and
98 pollen. As pollen recognition system, many Brassicasceae species have sporophytic SI
99 system. SI is a sophisticated system for pollen selectivity to prevent self-fertilization and is
100 genetically controlled by a single highly polymorphic *S* locus (Bateman 1995). *S*-locus
101 receptor kinase (SRK) and *S*-locus protein 11 (SP11)/*S* locus cystein-rich protein (SCR) have
102 been identified as female and male *S*-determinant factors, respectively (Stein et al. 1991,
103 Takasaki et al. 2000, Takayama et al. 2000, Schopfer et al. 1999). In addition, *M*-locus
104 receptor kinase (MLPK) and arm repeat containing 1 (ARC1) have been identified as
105 downstream signaling factors, interacting with SRK (Gu et al. 1998, Stone et al. 1999, Stone
106 et al. 2003, Murase et al. 2004, Kakita et al. 2007a, Kakita et al. 2007b). Exo70A1, a
107 subunit of the exocyst complex, is a target of ARC1and has been identified as a mediator of
108 water transport for pollen from papilla via vesicle trafficking (Samuel et al. 2009). The
109 *Brassica* SI response has been observed as prevention of pollen hydration and inhibition of
110 pollen tube penetration (Zuberi and Dickinson, 1985, Dickinson, 1995). Recently, it was
111 reported that pollen hydration was regulated by a balanced combination of multiple
112 components of hydration, rehydration and dehydration, through a time-series observation of
113 pollen behavior on pollination (Hiroi et al. 2013). Thus, pollen hydration is controlled by a
114 complex water transport system in papilla cell, and it has been predicted that vacuolar
115 dynamics in papilla is involved in water transport for pollen hydration (Iwano et al. 2007).

116 After pollen hydration, germinated pollen tube exactly penetrates into papilla cell, and it is
117 believed to be regulated by own cell wall degrading enzymes, triggering a tube elongation
118 between cell walls of papilla cell. Plantacyanin, a subfamily of blue copper proteins, has
119 been identified as a regulator of *in vitro* pollen tube reorientation in lily (Kim et al. 2003), and
120 it has been reported that pollen tube guidance at papilla cell was disrupted by over-expression
121 of plantacyanin in *A. thaliana* (Dong et al. 2005). Although, as stated above, many genes
122 and events in pollination have been revealed (reviewed in Suwabe et al. 2010, Watanabe et al.
123 2012), it is still impossible to explain the overall molecular systems of pollination, and
124 molecular factors involved in pollination are also still largely unclear. To overcome this
125 situation, transcriptome analysis is effective to establish a knowledge base to understand
126 molecular mechanisms and factors in pollination. Recently, we established the cell
127 type-specific transcriptome system in Brassicaceae papilla cells, by a combination of laser
128 microdissection and RNA sequencing (LM-RNA-seq) (Osaka et al. 2013). This method can
129 be widely applicable to various conditions and tissues, such as pollination step and
130 developmental stage, and thus functional analysis at transcription level in papilla cell should
131 become a beachhead to understand molecular systems during pollination.
132 In this study, we report the characterization of gene expression profiles among non-,
133 compatible- and incompatible-pollinated papilla cells in *A. thaliana*, by LM-RNA-seq
134 analysis. Using these papilla cell-specific gene expression profiles in pre- and post-

pollinations, we investigated molecular systems and factors involved in pollination. By bioinformatics analysis, biological characteristics and difference of papilla cell between pre- and post-pollinations, and compatible- and incompatible-pollinations, were also discussed.

Results and discussion

RNA sequencing analysis

To establish a list of genes expressed in papilla cell at pre- and post-pollination, total RNAs were extracted from non-pollinated (NP), compatible-pollinated (CP) and incompatible-pollinated (IP) papilla cells. Their acceptable quality for RNA-sequencing were confirmed by RNA integrity number (RIN), a quality value of total RNAs on a scale from 1 to 10; 7.6, 6.1 and 6.2 for NP, CP and IP papilla cells, respectively (Fig. 1). After liner amplification of mRNAs from extracted total RNAs, fragment libraries were subjected to sequencing on the Ion Proton platform. As a result of the RNA-sequencing, 74,572,833, 77,704,982 and 72,870,933 raw reads were obtained from NP, CP and IP papilla cells, respectively, and among them 95.33%, 96.46% and 93.99% were mapped to the *Arabidopsis* reference genome, respectively, on the alignment of raw reads to the *A. thaliana* genome sequences (TAIR10) (Table 1). Expected genes were calculated from mapped reads and more than 10 reads per at least one transcript isoform were defined as expressed genes. As a result, 15,955, 18,057 and 17,533 genes were found to be expressed in NP, CP and IP papilla

154 cells, respectively. Expressed genes were further classified into the protein coding gene,
155 pseudogene, transposable element gene and non-coding RNAs, and we regarded here the
156 protein coding gene and pseudogene as the papilla cell-expressed gene, because it is known
157 that it depends on the situation of ecotype, or line, whether some gene is functional or not in
158 each ecotype, e.g. pseudogene in Col-0 but functional gene in Old-1 for *SRK* (Shimizu et al.
159 2008, Tshuchimatsu et al. 2010). Consequently, 15,475, 17,360 and 16,918 genes were
160 identified as the papilla cell-expressed genes for NP, CP and IP papilla cells, respectively
161 (Table 2). These data are consistent with our previous estimation, in which approximately
162 17,000 genes are expressed in papilla cell of Brassicaceae (Osaka et al. 2013).
163 Among expressed genes in NP, CP and IP papilla cells, 14,392 genes were commonly
164 found in all data sets, corresponding to 93% of the expressed genes in NP, 82.9% in CP and
165 85.1% in IP papilla cells, and 374, 1,008 and 663 genes were specifically found in NP, CP and
166 IP papilla cells, respectively (Fig. 2). This result indicates that a majority of expressed genes
167 commonly function in pre-, post-, compatible- and incompatible-pollination. However,
168 because a direct comparison of expressed genes is not taken into account the basic
169 information of their expression level, and therefore we consider that direct comparison of
170 expressed genes is not appropriate for an investigation of functional change of genes between
171 pre- and post-pollination and also compatible and incompatible pollinations.
172

Identification of differentially expressed genes and Gene Ontology analysis

Functional change of papilla cell for pollen recognition, acceptance/rejection of pollen grain and pollen tube guidance is controlled by complex cellular systems which are regulated by a diversity of molecular players, although most of these are still largely unclear. Thus, to investigate the molecular basis of these functional changes during pollination, differentially expressed genes (DEGs) analysis was conducted for CP and IP against NP papilla cells (Fig. 3). First, in a comparison of DEGs, 278 up-regulated and 112 down-regulated genes were commonly detected among pre-, post-, compatible- and incompatible-pollinations, and totally 400 genes, which correspond to 2.3% of the expressed genes in CP and 2.4% in IP papilla cells, only showed their expression difference during pollination (Fisher's exact test, p-value=<0.05, fold change >=3). Second, 147 and 71 genes were up-regulated in CP and IP papilla cells, corresponding to 0.85% of the expressed genes in CP and 0.42% in IP, and 115 and 46 genes were down-regulated in CP and IP papilla cells, corresponding to 0.66% of the expressed genes in CP and 0.27% in IP. These results suggest that molecular factors contributing to pollination system in papilla cell have little difference between pre- and post-pollinations and the functional change of papilla cell on pollination depends on either of small number of genes or alteration of gene expression level.

To further investigate molecular and biological functions of DEGs, functional classification of DEGs were conducted by Gene Ontology (GO) analysis. The top 10 most

192 highly represented GO terms in each category of cellular components, molecular functions
193 and biological processes are shown in Fig. 4. In cellular components, GO terms categorized
194 in nucleus, plasma membrane and extracellular region were predominant in all DEGs (Fig.
195 4A). In a category of nucleus, transcription factor (TF) genes were also included: it is
196 known that MYB, one of typical TFs, family genes are required for pollen tube and synergid
197 cell differentiations for successful fertilization in *A. thaliana* (Kasahara et al. 2005, Lydon et
198 al. 2013). In a category of plasma membrane, GO term "protein kinases", such as protein
199 kinase superfamily protein and calcium-dependent protein kinase, were mostly represented as
200 up-regulated genes, compared with down-regulated genes. In a category of extracellular
201 region, GO term "small peptide" such as rapid alkalinization factor (RALF) and secretory
202 small cysteine rich protein (CRP), functioning in signal transduction, were predominant. In
203 plant reproductive processes, signal recognition and transduction through interaction between
204 CRP and protein kinase, a.k.a. receptor-like kinase (RLK), are critical in many steps, such as
205 pollen recognition, pollen tube guidance and fertilization. The fact that such molecular
206 factors categorized in plasma membrane and extracellular region are predominant in papilla
207 cell indicates that information exchange with pollen and extra-environment actively function
208 in papilla cell, and regulation of these gene expression are one of keys in successful
209 reproduction, i.e. pollen-pistil interaction, and maintenance/protection itself from external
210 attacks and cues.

211 The predominant GO terms in molecular functions were “zinc ion binding” and
212 “calcium ion binding” (Fig. 4B). In “zinc ion binding”, some were specifically up-regulated
213 in CP and others were down-regulated both in CP and IP papilla cells. Zinc ion is essential
214 for stabilization of E3 ubiquitin ligase, in which the RING (really interesting new gene) finger
215 domain is essential for ubiquitination of target protein for protein degradation by proteasome
216 (Smalle and Vierstra 2004). There is an example of active protein degradation by
217 proteasome in pollination. In *Brassica* self-incompatibility system, ARC1, one of the E3
218 ubiquitin ligase, has been identified as interacting with SRK kinase domain in *B. napus* (Ref).
219 Exo70A1, a member of exocyst subunit family, is a target of ARC1 and mediates
220 Golgi-derived vesicles to target membranes to transport water for pollen grains (Ref). In
221 compatible pollination, ARC1 is not active and Exo70A1 can supply water to pollen from
222 papilla cell by exocytosis. By contrast, in incompatible pollination, ARC1 activated by SRK
223 degrades Exo70A1 by proteasome system and water supply is aborted, leading to pollen
224 rejection. Thus, water transport system from papilla to pollen is regulated through a
225 down-regulation of ARC1 and up-regulation of Exo70A1 in pre-pollination state, also in
226 compatible pollination state, and, once incompatible pollination is initiated, ARC1 is
227 positively activated for inhibition of water supply from papilla cell to pollen, through
228 ubiquitination and degradation of Exo70A1. In “calcium ion binding”, calcium-binding
229 EF-hand family proteins and calcium-dependent protein kinase were up-regulated in all DEGs

(Fig. 4C). These up-regulated genes would act as Ca^{2+} signaling in papilla cell. Together with the fact that concentration of cytoplasmic Ca^{2+} increases at the pollen tube germination site and tip region after compatible pollination (Iwano et al. 2004), it is clear that cytoplasmic Ca^{2+} is one of key factors for a cellular process both in male and female reproductive functions.

In addition to the above consideration, it is noteworthy that GO terms involved in "transferase activity", "catalytic activity", "metabolic process" and "oxidation-reduction process" were highly represented in all DEGs, in molecular functions and biological processes (Fig. 4C). This result indicates that various metabolic and biosynthesis pathways are actively involved in compatible and incompatible pollinations. Therefore, we conducted pathway analysis of DEGs to investigate detailed function in pollination, at metabolic pathway.

Pathway analysis of DEGs

To characterize metabolic pathways working in papilla cell during pollination, DEGs were mapped to metabolic pathways using KEGG mapper tool (Kyoto Encyclopaedia of Genes and Genomes: KEGG). No mapped DEGs were annotated KEGG Orthology (KO) numbers by Blast KOALA and also mapped to metabolic pathways. Among them, 97 DEGs were mapped to various metabolic pathways, and they were categorized into 7 metabolic pathways

249 (Fig. 5). Genes belonging to carbohydrate metabolism was the most observed in
250 up-regulated genes in CP and IP papilla cells, and especially they were found in starch and
251 sucrose metabolism pathway and pentose and glucuronate interconversions pathway (Fig. 6).
252 β-glucosidase (EC 3.2.1.21) presented in down-regulated genes in CP papilla cell, and
253 invertase (EC 3.2.1.26) presented in up-regulated genes in both pollinations. These enzymes
254 are involved in α-/β-D-glucose biosynthesis (Ketudat Cairns and Esen 2010, Fotopoulos
255 2005). Genes related to trehalose biosynthesis, from UDP-glucose, were down-regulated in
256 both pollinations. In galactose metabolism pathway, UDP-D-glucose/UDP-D-galactose
257 4-epimerase (EC 5.1.3.2) was up-regulated in CP and down-regulated in IP papilla cells.
258 β-galactosidase 2 (EC 3.2.1.23), a catalytic enzyme for hydrolysis of lactose into D-galactose
259 and α-D-glucose (Gantulga et al. 2009), were down-regulated in IP papilla cell. These
260 results indicate that sugar metabolism actively functions in both CP and IP papilla cells, and
261 glucose and UDP-glucose seems to be accumulated in papilla cell after pollination in
262 compatible pollination. Glucose-specific monosaccharide transporters, AtSTP9 and
263 AtSTP11, were identified to function in pollen tube in *A. thaliana* (Schneidereit et al. 2003,
264 2005), and in our data some genes related to sugar transport were up-regulated in CP papilla
265 cell and others were down-regulated in IP papilla cell. UDP-glucose is a source of callose,
266 essential material of pollen tube wall and callose plug in pollen tube (Schlupmann et al. 1994,
267 Nishikawa et al. 2005). Growing pollen tube requires an optimal supply of nutrition from

surrounding female tissues for their activity, because pollen is basically inactive sink for photosynthesis and it is impossible to produce carbohydrates as an energy source by itself. Thus, cooperation of sugar production/metabolism in papilla cell and export to pollen by transporter is critical for pollen activity in reproduction process, and they would be regulated in both processes. In addition, a part of up-regulated sugar metabolic pathways and transporters in CP papilla cell were down-regulated specifically in IP papilla cell. Thus, pollen acceptance/rejection on papilla cell for pollination may be regulated by alteration of energy source condition for pollen tube growth, via sugar metabolism and transport systems.

Another represented sugar metabolic pathway was associated with pectin and cellulose metabolism (Fig. 6). Genes related to pectin and cellulose degradation pathways were up-regulated in CP papilla cell, and pectin esterase (EC 3.1.1.11), synthesizing pectate by hydrolysis of pectin, was up-regulated in IP and down-regulated in CP papilla cells. The major part of plant cell wall is composed of proteins and polysaccharides such as pectin, cellulose and hemicellulose (Cosgrove 2005, Lehner et al. 2010). In Brassicasceae, after pollen recognition, pollen hydration and pollen germination, pollen tube penetrates the outer layer of cell wall of papilla cell and grows in the apoplastic space down to the ovary to deliver sperm cells for successful fertilization. In this process, cell wall of papilla is softened and expanded prior to pollen tube penetration, and pectinase and expansin, functioning in cell wall modification, in papilla cell should contribute for loosing of papilla cell wall. It has been

287 considered that penetration of pollen tubes into papilla cell is controlled by the cell wall
288 modifying enzymes, such as pectinase and polygalacturonase, which are derived from pollen
289 (Dearnaley and Daggard 2001). On the contrary, in *B. napus*, it was reported that serine
290 esterases, identified in pollen tube and stigma, are required for pollen tube penetration into
291 papilla cell (Hiscock et al. 2002). In tobacco, a pistil-specific β-expansin, PPAL, is secreted
292 in the stigmatic exudate and acts as a cell wall-loosening (Pezzotti et al. 2002). Therefore,
293 from our result and previous reports, loosing of papilla cell wall for pollen tube penetration
294 would be controlled by cell wall modifying enzymes derived from papilla cell itself, in
295 addition to derived from pollen. Activation of cell wall remodeling of papilla cell would be
296 one of differences between CP and IP papilla cells: degradation in CP papilla cell for
297 acceptance of pollen tube and composition in IP papilla cell for rejection of pollen. In
298 connection with this, genes related to secondary metabolites of phenylpropanoid biosynthesis
299 were also represented in up-/down-regulated genes in CP papilla cell (Fig. 7). Caffeoyl
300 coenzyme A O-methyltransferase 1 (CCOAOMT1; EC 2.1.1.104) and O-methyltransferase 1
301 (OMT1; EC 2.1.1.68), involving in lignin biosynthesis, were down-regulated in CP papilla
302 cell. Peroxidase superfamily genes (EC 1.11.1.7) were found to be up- and down-regulated
303 in CP papilla cell, and they also mediate lignin biosynthesis that metabolizes lignin from
304 monolignol. Lignin, a phenolic macromolecular compound, is a major component ensuring
305 strong lignification of plant tissue, and thus these results suggest that balanced cell wall

306 remodeling of papilla cell, via pectin and cellulose metabolism and lignin biosynthesis, is
307 important element in successful pollination, especially in pollen tube reception/rejection.
308
309 **Conclusion**
310 In this study, we characterized transcriptional difference in papilla cells between pre- and
311 post-pollinations, and compatible and incompatible pollinations. Molecular and biological
312 function of papilla cell at pollination was also estimated via DEG analysis and metabolic
313 process analysis. They show characteristic feature of papilla cell as a female reproductive
314 organ in pollination, and it has variety of cellular systems: information exchange with pollen
315 and external environment, signal transduction through ligand-receptor interaction, internal
316 physiological change for water and nutrition supply to pollen, and external morphological
317 modification to accept/reject pollen grains. They are regulated by a diversity of molecular
318 players and sophisticated/well-orchestrated system, as an early step for successful
319 reproduction. The wide variety of genes are definitely important to ensure such correct
320 cellular, physiological, developmental and reproductive processes in papilla cells, and, from
321 our transcriptome data, they would be regulated by an alteration of gene expression level, not
322 the presence or absence of transcription.
323
324 **Materials and Methods**

325 **Plant materials**

326 *A. thaliana* ecotype Oldenburg (Old-1) and transgenic self-incompatible Old-1 (Matsuda et al.,
327 manuscript in preparation) were grown in a growth chamber with 8 h light/16 h dark cycle at
328 22°C. Old-1 is self-compatible, categorized as A-t4 haplotype (Shimizu et al 2008), but still
329 retains the female SI function, although the male SI gene *SCR* is already disrupted
330 (Tsuchimatsu et al 2013). Thus, pistils of wild type Old-1 is capable of analyzing female
331 part on compatible and incompatible pollination reactions. For hand pollination, flower buds
332 of wild type Old-1 were emasculated at developmental stage 12 (Smyth et al., 1990) and
333 incubated overnight at 22°C on 1% ager medium. Emasculated wild type pistils at
334 developmental stage 14 were pollinated with pollen either of wild type Old-1 (compatible
335 pollen donor) or transgenic self-incompatible Old-1 (incompatible pollen donor). At 1h after
336 pollination, pollinated pistils were collected and immediately fixed by ethanol/acetate 3:1
337 (v/v) solution on ice. Non-pollinated pistils at developmental stage 14 were collected and
338 were placed immediately in ethanol/acetate 3:1 (v/v) fixative solution on ice.

339

340 **Preparation of paraffin-embedded sections**

341 Tissue fixation, paraffin embedding and paraffin-embedded sections were conducted
342 according to methods of Osaka et al (2013). Briefly, fixed samples of non-pollinated,
343 compatible pollinated and incompatible pollinated pistils were dehydrated by serial dilutions

of 70, 80, 90, 100% and absolute ethanol, by using a microwave processor (LabPulse H2850, Energy Beam Sciences, East Granby, CT, USA). For paraffin-embedding, each fixative was replaced by 50% paraffin/50% 2-propanol by microwaving, and samples were then embedded into paraffin wax (Paraplast X-TRA, Fisher Scientific, Houston, TX, USA). The embedded tissue specimens were cooled to room temperature, and the paraffin blocks were stored at 4°C. The paraffin-embedded tissues were cut into 6 μm-thick sections using microtome (RV240, YamatoKoki, Saitama, Japan), within 1 day from paraffin embedding. Serial paraffin sections were mounted on PEN membrane frame slides (Life Technologies, Carlsbad, CA, USA), with RNAsecure Reagent (Ambion, Life Technologies) diluted in approximately 25 times by nuclease free water (Invitrogen, Life Technologies), and spread onto a heating plate at 57°C for 30sec to 1 min. One PEN membrane frame slide was used per 2 paraffin embedded pistils. After removing RNAsecure Reagent from PEN membrane frame slides using RNase free paper, paraffin sections were dried and stored at 4°C for at least 1 h.

Isolation of papilla cell, extraction of total RNAs and sample preparation for RNA sequencing

For total RNA extraction, papilla cells were collected from paraffin sections of non-pollinated, compatible pollinated and incompatible pollinated pistils, using laser microdissection system (Arcturus XT Laser Capture Microdissection System, Applied Biosystems, Life Technologies).

363 Extraction and quality check of total RNAs and linear-amplification of mRNAs were
364 conducted according to Osaka et al (2013). Total RNAs were extracted from 4-6 paraffin
365 embedded pistils for each sample and assessed their quantity and quality using an Agilent
366 2100 Bioanalyzer and RNA 6000 Pico kit (Agilent Technologies, Santa Clara, CA, USA),
367 with Eukaryote Total RNA Pico parameter. Total RNAs with a quality value of more than
368 RIN=6 were applied to linear-amplification, by a RiboAmp HS PLUS RNA amplification kit
369 (Life Technologies).
370
371 **RNA sequencing**
372 After amplification of cDNAs by 5 cycles of PCR, preparation of a fragment library for Ion
373 torrent sequencing was carried out following the manufacturer's instructions (Life
374 Technologies), using the Ion Plus Fragment Library Kit for AB Library Builder System (Life
375 Technologies) and AB Library Builder System (Life Technologies). Preparation of cDNA
376 fragment library was carried out by the Ion One Touch 2 System using the Ion PGM Template
377 OT2 200 Kit (Life Technologies). Prepared libraries were subjected to sequencing by the
378 Ion Proton platform, using Ion PI Sequencing 200 Kit v3, with one Ion Proton I Chip per each
379 sample. Sequence reads were aligned to the *A. thaliana* genome sequences (TAIR10) by Ion
380 Torrent Suite Software 4.2.1 (Life Technologies), with RNA-seq parameter.
381

Sequence data processing and differentially expressed genes analysis

After aligning, the resulting BAM files were sorted by SAMtools 0.1.19 (Li et al. 2009). Sorted BAM file were imported into Partek Genomics Suite version 6.6 (Partek, St. Louis, MO, USA) and calculated the mapped reads to genomic region using *A. thaliana* annotation file (TAIR10), GFF3 format file downloaded from Ensembl plants (http://plants.ensembl.org/index.html). Read counts per gene locus were calculated from reads mapped to the genome and normalized their expression value by reads per kilobase of exon per million mapped reads (RPKM). To identify differentially expressed genes (DEGs) between pre- and post-pollinations, statistical analysis was carried out using Fisher's exact test by R 3.01 software. We defined the DEGs at p-value=<0.05 and RPKM of more than 3-fold expression change. Functional categorization of DEGs by Gene ontology (GO) was performed using GO annotations from the TAIR website (http://www.arabidopsis.org/). In annotation of GO for DEGs, GO terms were confirmed and categorized by GO evidence codes, and GO terms Inferred from the Reviewed Computational Analysis and the Non-traceable Author Statement were removed. Metabolic pathway analysis of DEGs was carried out based on Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/), and DEGs were mapped onto metabolic pathways using KEGG Mapper tool. Non mapped DEGs were annotated KEGG Orthology (KO) numbers by Blast KOALA and mapped to metabolic pathways based on KO number.

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Legends to figures

Fig. 1 Quality of total RNAs of isolated papilla cells. Total RNAs were extracted from (A) non pollinated (NP), (B) compatible pollinated (CP) and (C) incompatible pollinated (IP) papilla cells. Quality of total RNAs was evaluated by RNA Integrity Number (RIN) using an Agilent 2100 Bioanalyzer.

Fig. 2 Comparison of papilla-expressed genes between pre- and post-pollinations. Venn diagram for the number of genes in papilla cells from NP, CP and IP papilla cells.

Fig. 3 differentially expressed genes (DEGs) between pre- and post-pollinations. Genes are classified according to comparison between CP and IP papilla cells. The number of up-regulated (pink) or down-regulated (blue) genes are shown in the bar chart.

Fig. 4 Functional classification of DEGs between pre- and post-pollinations. Six groups of differentially expressed genes in compatible, incompatible and both pollinations were classified into 3 Gene Ontology (GO) categories, (A) cellular components, (B) molecular functions and (C) biological processes. Right and left bar charts indicate number of up-regulated and down-regulated genes, respectively. Number of DEGs are shown by 6 color bar charts, (red) up-regulated in both pollinations, (blue) up-regulated in compatible

pollination, (green) up-regulated in incompatible pollination, (purple) down-regulated in both pollinations, (orange) down-regulated in compatible pollination and (gray) down-regulated in incompatible pollination.

Fig. 5 Classification of metabolic pathways in DEGs between pre- and post-pollination. Metabolic pathways were classified into 7 categories, (orange) carbohydrate metabolism, (blue) energy metabolism, (pink) lipid metabolism, (green) amino acid metabolism, (yellow) glycan biosynthesis and metabolism, (red) metabolism of cofactors and vitamins and (purple) biosynthesis of other secondary metabolites. Upper and lower bar charts indicate number of up-regulated and down-regulated genes, respectively.

Fig. 6 Change of sugar metabolic pathways between pre- and post-pollinations. Pathway maps of starch and sucrose metabolism, galactose metabolism and fructose and mannose metabolism are partially extracted from the KEGG pathways and merged. Black arrow indicates the pathway not mapped in DEGs. DEGs mapped pathways are shown in 6 color arrows, (red) up-regulated in both pollinations, (purple) up-regulated in compatible pollination, (blue) down-regulated in both pollinations, (yellow) down-regulated in compatible pollination.

635 **Fig. 7** Change of phenylpropanoid biosynthesis pathways between pre- and post-pollinations.
636 Pathway maps are partially extracted from the KEGG pathways. Black arrow indicates
637 pathways not mapped in DEGs. DEGs mapped pathways are shown in 5 color arrows, (red)
638 up-regulated in both pollinations, (purple) up-regulated in compatible pollination, (green)
639 up-regulated in incompatible pollination, (blue) down-regulated in both pollinations and
640 (yellow) down-regulated in compatible pollination.

641

642 **Supplementary data**

643 **Supplementary Table S1** List of DEGs between pre- and post-pollinations.

644

645 **Supplementary Table S2** List of DEGs mapped in metabolic pathway.

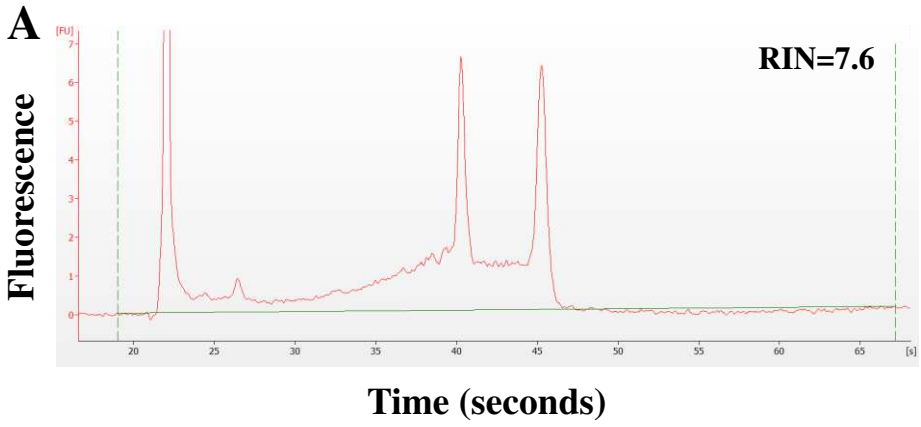


Fig. 1 Matsuda et al.

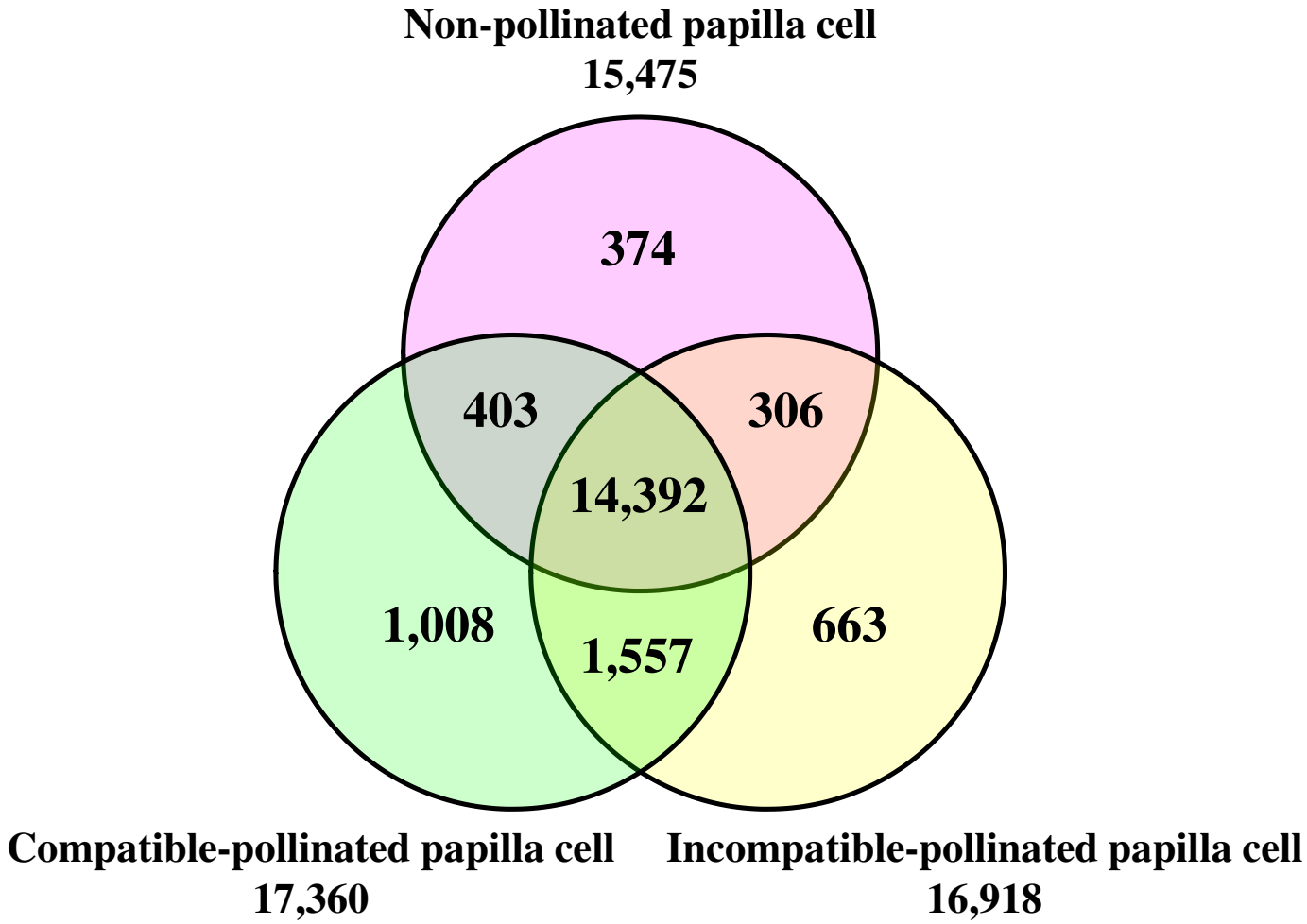


Fig. 2 Matsuda et al.

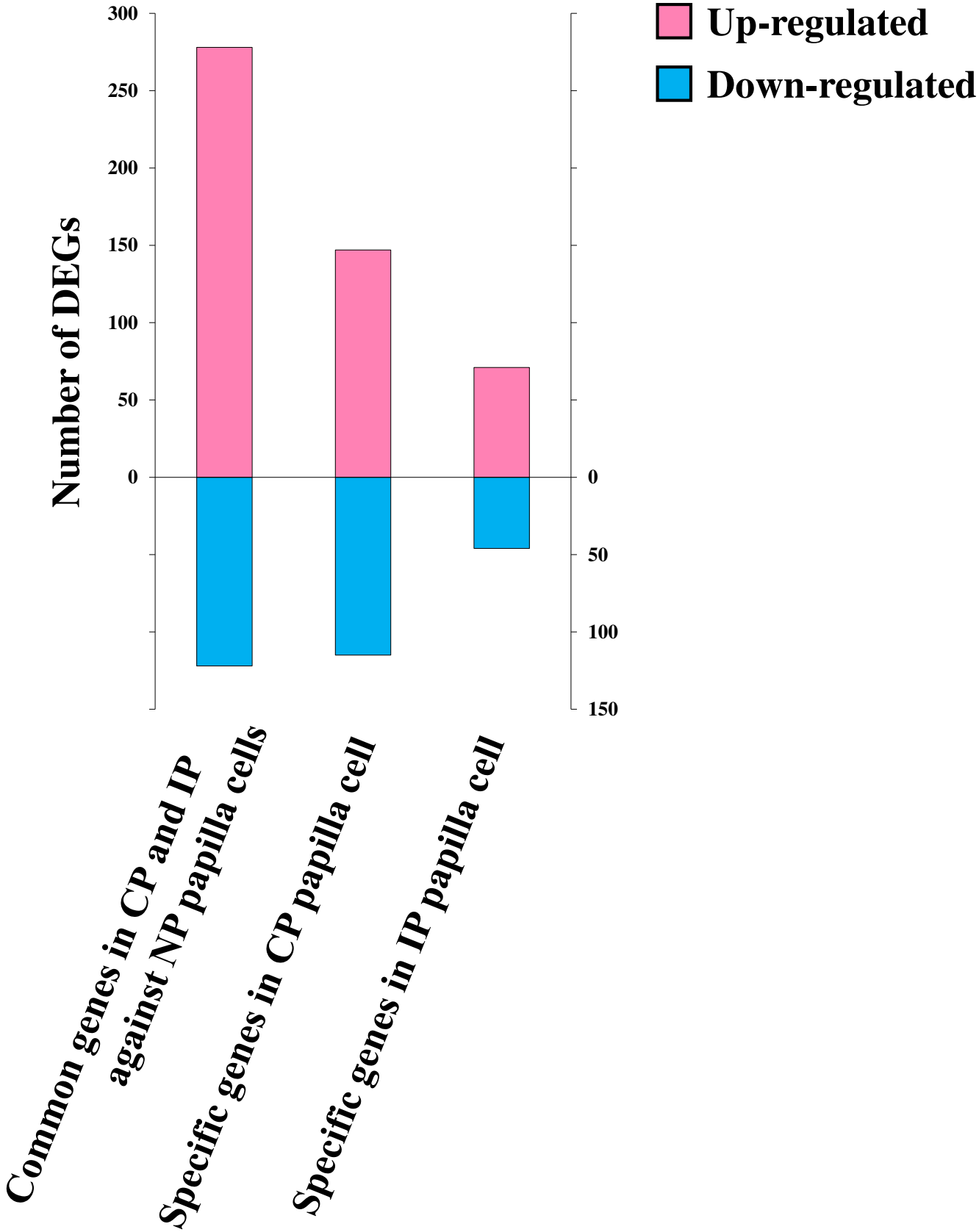
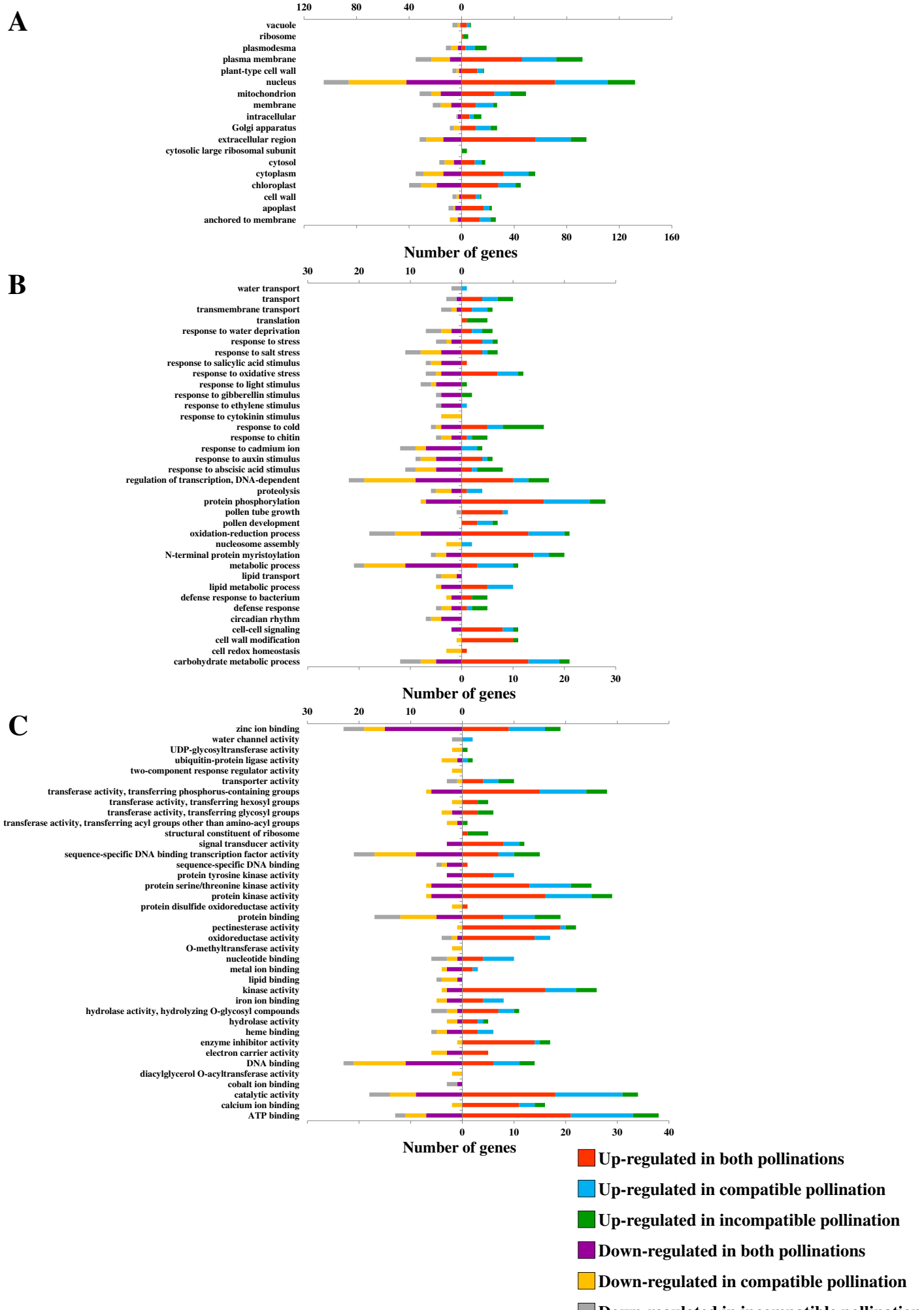


Fig. 3 Matsuda et al.



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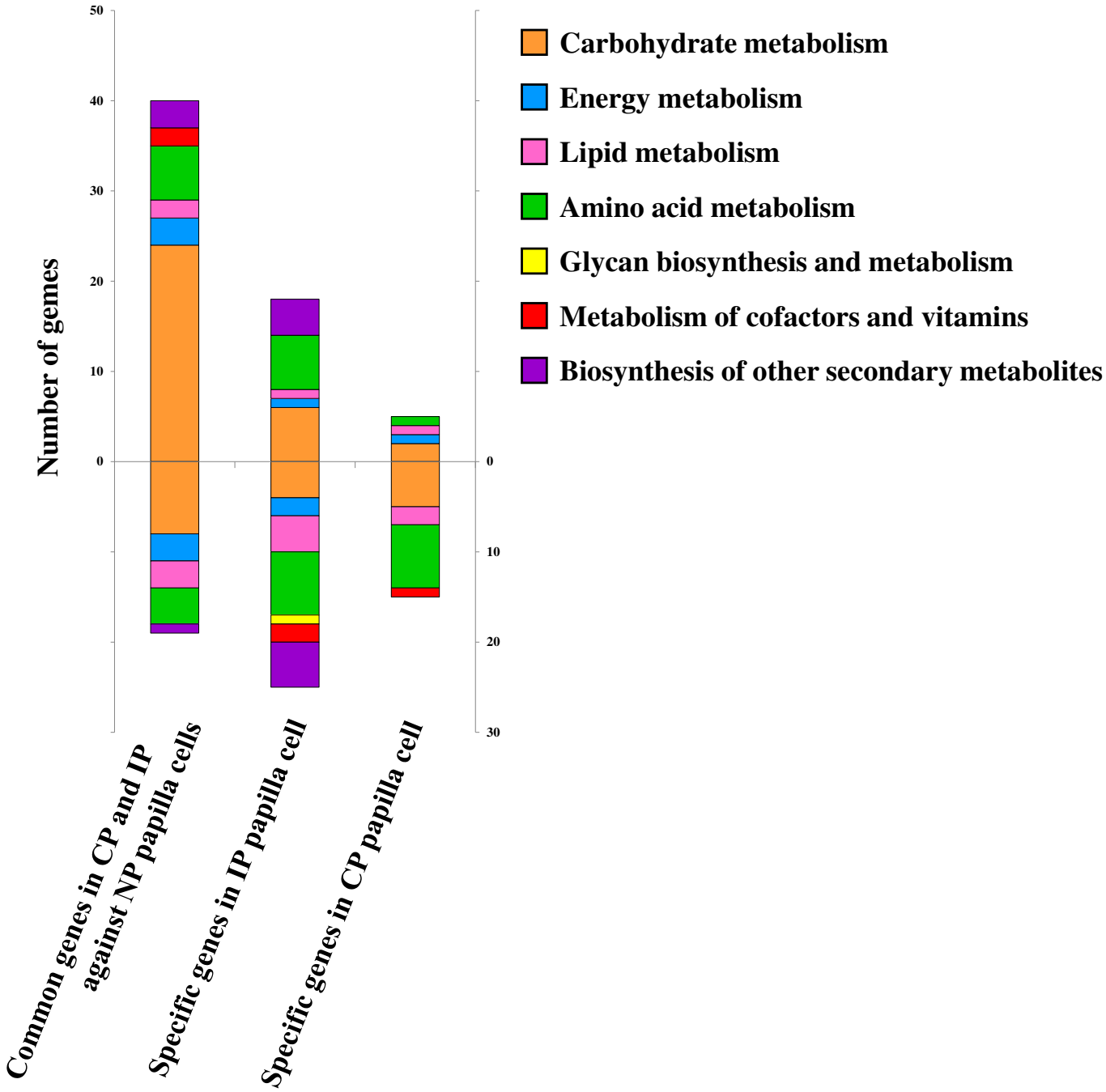

Fig. 5 Matsuda et al.

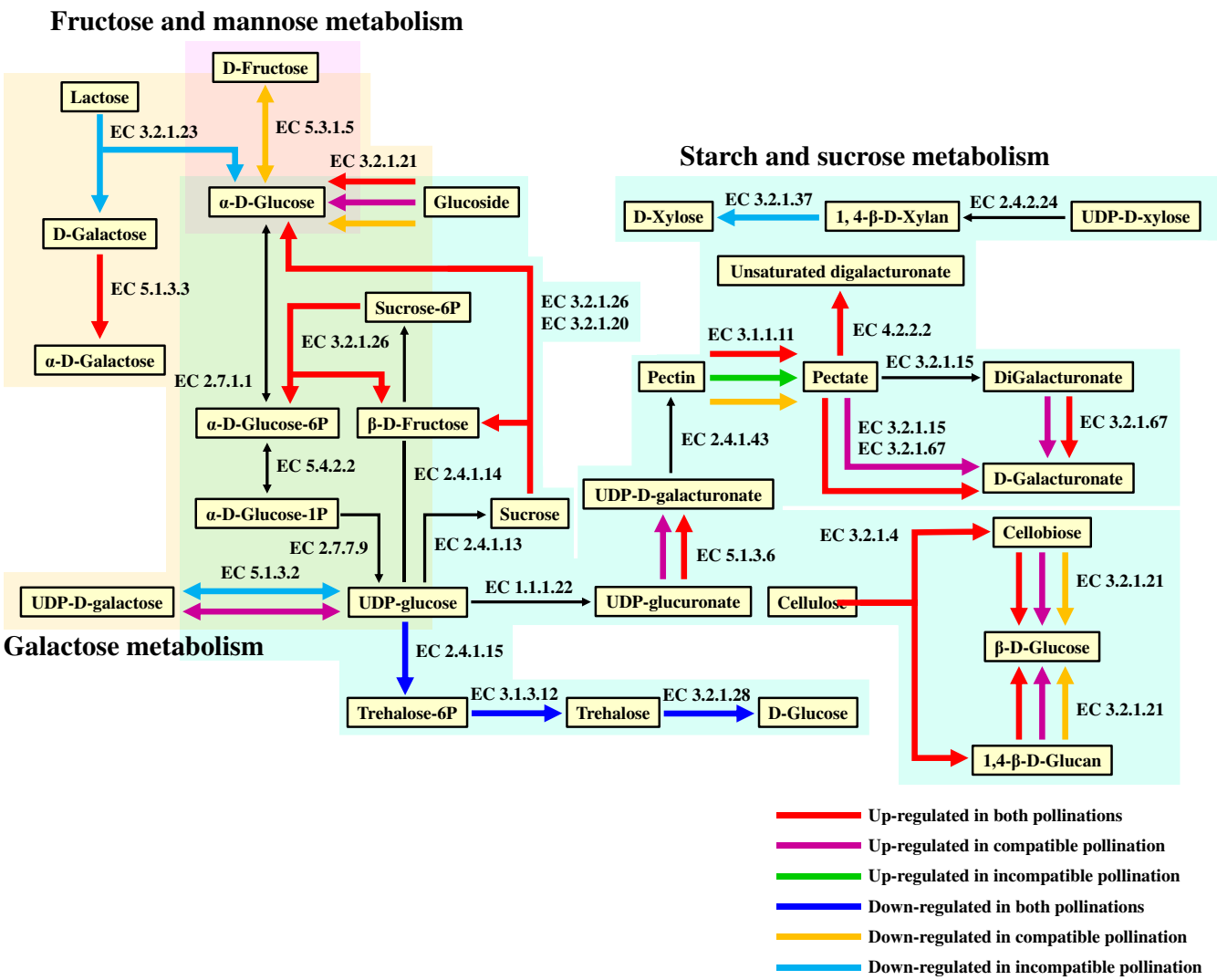

Fig. 6 Matsuda et al

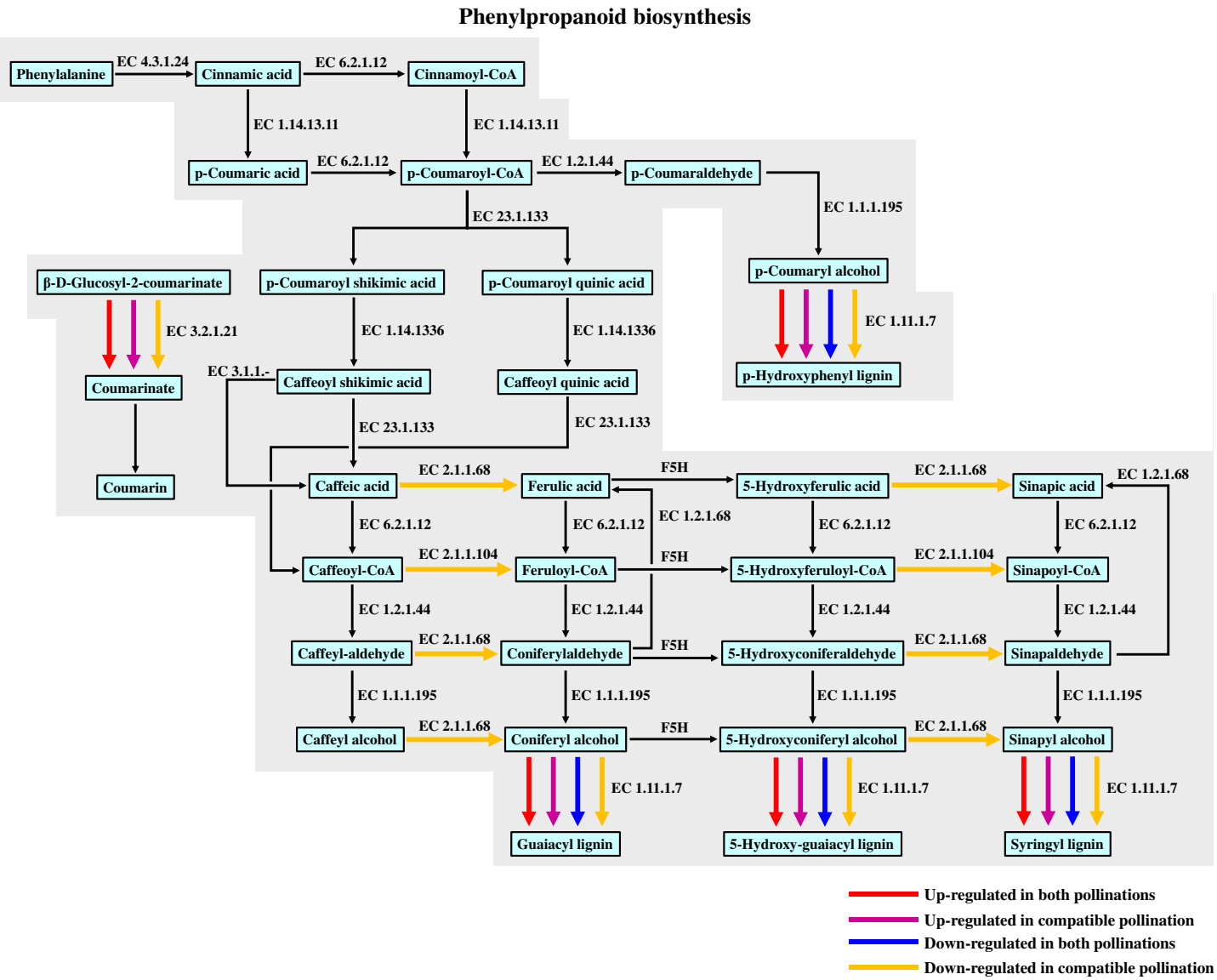

Fig. 7 Matsuda et al

Table 1 Results of RNA-sequencing in non-, compatible- and incompatible-pollinated papilla cells

Type of papilla cell	Total reads	Mapped reads to genome	Genes
Non-pollinated	74,572,833	67,223,751	15,955
		95.33%[a]	47.48%[b]
Compatible pollinated	77,704,982	63,376,799	18,057
		96.46%[a]	53.74%[b]
Incompatible pollinated	72,870,933	71,581,421	17,533
		93.99%[a]	52.18%[b]

a: Percentage of mapped reads to genome per total reads

b: Percentage of genes per total genes in *A. thaliana* (33,602 genes)

Table 2 Categorization of papilla cell expressed genes in non-, compatible- and incompatible-pollinated papilla cells

Type of papilla cell	Protein coding genes	Pseudogenes	Transposable element genes	Non-coding RNAs
Non-pollinated	15,356	119	287	193
Compatible pollinated	17,209	151	468	229
Incompatible pollinated	16,782	136	413	202